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Kinetic Enzymatic Method for Automated Determination of HDL Cholesterol in Plasma

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Summary: A sensitive fixed-time kinetic enzymatic method for the measurement of high density lipoprotein cholesterol is described, as adapted for the Cobas Bio centrifugal analyser (Hoffmann LaRoche). The Boehringer Mannheim cholesterol esterase/cholesterol oxidase/peroxidase/3,4-dichlorophenol kinetic reagent was modified by the inclusion of 2,4,6-tribromo-3-hydroxybenzoic acid which reacts with hydrogen peroxide and 4-aminophenazone to produce a quinone-imine dye with a greater molar absorptivity than that produced with phenol. The method has been developed for the determination of HDL fractions isolated with polyethylene glycol 6000, for which a reagent of high sensitivity is required.

The method is linear to at least 3.88 mmol/l of HDL cholesterol and the coefficients of variation for within-run and day-to-day precision were less than 3.0%. Correlation of the kinetic HDL cholesterol method with an equilibrium method was good (R = 0.9980).

The assay is rapid, inexpensive and large numbers of specimens can be processed conveniently.

Introduction

The analysis of high density lipoprotein cholesterol has, in epidemiological and clinical studies (1–6), shown that a low HDL cholesterol level usually indicates a risk for coronary heart disease. The increased demand for HDL cholesterol assays had led to the need for a method capable of handling large numbers of assays generated from clinical and epidemiological work. The Boehringer Mannheim cholesterol oxidase 4-aminophenazone (CHOD-PAP) reagent for cholesterol has been adapted for use with a centrifugal analyser to measure HDL cholesterol as an equilibrium method (7). The incorporation of 3,4-dichlorophenol in the Boehringer Mannheim CHOD-PAP reagent has enabled the assay of total serum cholesterol using automated fixed-time kinetic methodology (8). The kinetic cholesterol method has considerably reduced the analytical time required, therefore allowing more efficient use of automated instruments, and has the advantage of being less sensitive to interferences than are equilibrium methods.

However, the adaptation of the kinetic method for the analysis of HDL cholesterol would involve the measurement of significantly smaller absorbance differences (ΔA). Since a relatively high photometric error would be incurred in the measurement of such low absorbance values (9), the assay was modified by adding 2,4,6-tribromo-3-hydroxybenzoic acid to the Boehringer Mannheim kinetic cholesterol reagent. The tribromohydroxybenzoic acid reacts with hydrogen peroxide and the 4-aminophenazone/peroxidase system to produce quantitative amounts of quinone-imine dye. Details of the modified assay are reported.

Materials and Methods

Apparatus

Cobas Bio centrifugal analyser (F. Hoffman-La Roche and Co., Limited Company, Basle, Switzerland) and related accessories. Hettich Universal K2S refrigerated centrifuge (Andreas Hettich, Tuttlingen, W. Germany).

Reagents

Buffered polyethylene glycol 6000: Polyethylene glycol, average relative molecular mass M_r = 6000 (British Drug Houses) 200 g/l in glycine/NaOH buffer, 0.2 mol/l, pH 10.

2,4,6-Tribromo-3-hydroxybenzoic acid: Synthesised by bromination of Analar grade 3-hydroxybenzoic acid (10).

Cholesterol reagent: Boehringer Mannheim "CHOD-PAP" kinetic colorimetric test (catalogue number 692905).

Cholesterol standards: Preciset® High Performance standards (Boehringer Mannheim, GmbH catalogue number 709905).

High Density Lipoprotein Cholesterol Control: Boehringer Mannheim Precinorm®L (lyophilised) catalogue number 781827, lot number 154747.

Procedures

Isolation of HDL

Buffered polyethylene glycol 6000 solution (0.2 ml) was mixed with plasma (0.2 ml). After 10 min at 20–25 °C, the mixture was centrifuged at 2000 *g* for 20 min at 4 °C. The supernatant was aspirated for cholesterol analysis. Positive displacement pipettes were used for all quantitative pipetting procedures.

Cobas Bio reagent boat

Equal quantities of 9 g/l NaCl solution and polyethylene glycol 6000 solution were mixed and placed in standard well 1. Cholesterol standards were mixed with equal quantities of polyethylene glycol 6000 solution. Boehringer Mannheim Preciset standard 1.29 mmol/l was placed in standard well 2, and standard 2.59 mmol/l in well 3. For 70 assays, the reconstituted CHOD-PAP reagent (25 ml) was combined with 2,4,6-tribromo-3-hydroxybenzoic acid and placed in the main well. This reagent was discarded after use. Various concentrations of 2,4,6-tribromo-3-hydroxybenzoic acid ranging from 0–20 mmol/l were evaluated to determine the most suitable strength.

Cobas Bio procedure

Various plasma/reagent ratios were tried in a series of preliminary experiments and the most suitable was found to be 1 to 55. This ratio and the optimum 2,4,6-tribromo-3-hydroxybenzoic acid concentration was a compromise to obtain the desired sensitivity required for a rapid kinetic method. The maximum absorbance of the quinone-imine dye, which represents the final product of the reaction, was at 515 nm. Absorbances measured in this region are less likely to be affected by colorimetric interference due to haemolysed or icteric plasmas. The instrument was operated according to the settings listed in table I.

Tab. 1. Parameter listing for determination of high density lipoprotein cholesterol on the Cobas-Bio centrifugal analyser by the fixed-time kinetic method.

Units	mmol/l
Calculation factor	0
Standard 1 conc.	0.0 (Polyethylene glycol blank)
Standard 2 conc.	1.29
Standard 3 conc.	2.59
Limit (mmol/l)	4.0
Temperature °C	30
Type of analysis	5
Wavelength (nm)	515
Sample Volume (µl)	15
Diluent Volume (µl)	30
Reagent Volume (µl)	350
Incubation time (s)	30
Start reagent volume (µl)	0
Time of First Reading (s)	10
Time interval (s)	10
Number of Readings	6
Blank mode	1 (reagent blank)

Results

Concentration of 2,4,6-tribromo-3-hydroxybenzoic acid

The concentration of 10 mmol/l 2,4,6-tribromo-3-hydroxybenzoic acid in the Boehringer Mannheim reagent produced the largest absorbance differences (ΔA) for a total reaction time of 50 seconds. These absorbance differences obtained using 2,4,6-tribromo-3-hydroxybenzoic acid were three times greater than those produced with phenol alone in the unmodified CHOD-PAP kinetic cholesterol reagent (fig. 1). Subsequent work was carried out using 10 mmol/l. 2,4,6-tribromo-3-hydroxybenzoic acid.

Course of the reaction

The feasibility of an assay protocol based on first-order kinetics was examined by following the progress curve of four aqueous cholesterol standards (0.65, 1.29, 2.59 and 3.88 mmol/l) and two patient plasmas (0.80 and 1.95 mmol/l HDL cholesterol), all treated with polyethylene glycol. The semilogarithmic plot of absorbance at completion of reaction minus absorbance at time t versus t indicates that the reaction follows first-order kinetics since the plot is linear from 10 seconds to at least four minutes (fig. 2). The initial lag phase is terminated within the first 10 seconds of the reaction. A total reaction time of 60 seconds provides sufficient linearity and a short analysis time. Therefore, the subsequent kinetic protocol was based on the preincubation of the 2,4,6-tribromo-3-hydroxybenzoic acid modified reagent at 30 °C for 30 seconds, the absorbance difference being calculated from the difference between the absorbances at 60 seconds and 10 seconds after plasma and reagent mixing (ΔA/50 seconds).

Linearity

Linearity was determined with the Preciset® cholesterol standards and their dilutions (fig. 3) and serial dilutions of a patient plasma with a HDL cholesterol value of 2.45 mmol/l (fig. 4). The linear range for the

standards was from at least 0.25 to 3.88 mmol/l. The ΔA values for the dilutions of the patient plasma were proportional within the dilution range tested (1 : 1 to 1 : 16). The linear range of the assay therefore covers the majority of the population values likely to be encountered (0.5 to 2.5 mmol/l) (7).

Accuracy and precision

Comparison of 48 patient HDL cholesterol plasma values obtained for a fixed-time kinetic method (x) and an equilibrium method (y) (7) showed good correspondence of results ($r = 9980$, $y = 1.011x + 0.016$) (fig. 5).

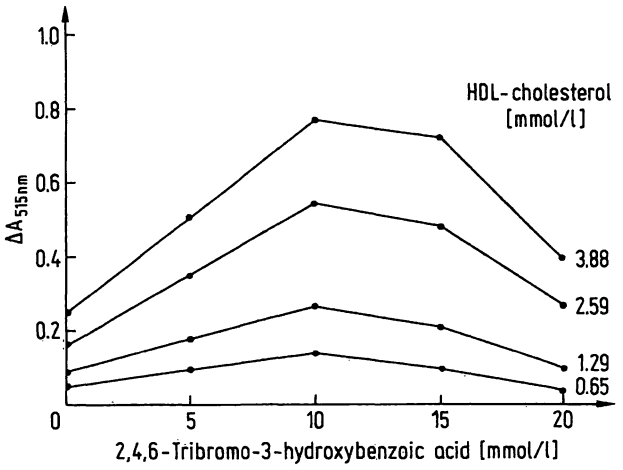

Fig. 1. Absorbance plots of HDL cholesterol standards utilising varying concentrations of 2,4,6-tribromo-3-hydroxybenzoic acid in the enzymic cholesterol reagent.

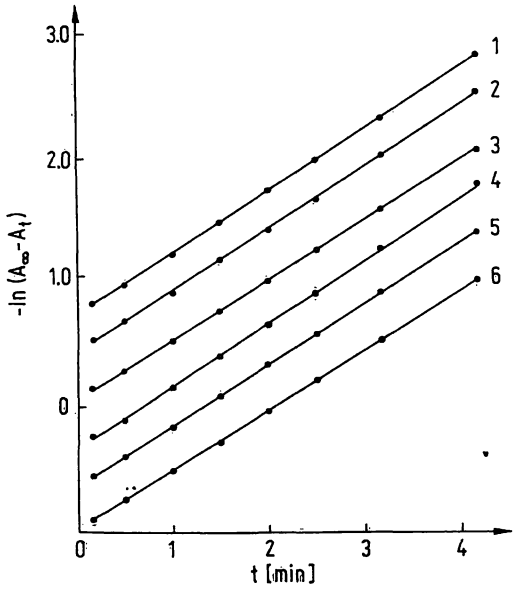

Fig. 2. Time course of reaction.
A_t, absorbance at time t; A_∞, absorbance after completion of reaction; rate constant, $k = -\Delta\ln(A_\infty - A_t)/\Delta t$ (slope of curves). Specimens: 1, 3, 5, 6, Preciset Cholesterol high performance (0.65, 1.29, 2.59, 3.88 mmol/l, respectively); 2, 4, human plasma (0.85, 1.99 mmol/l HDL cholesterol, respectively).

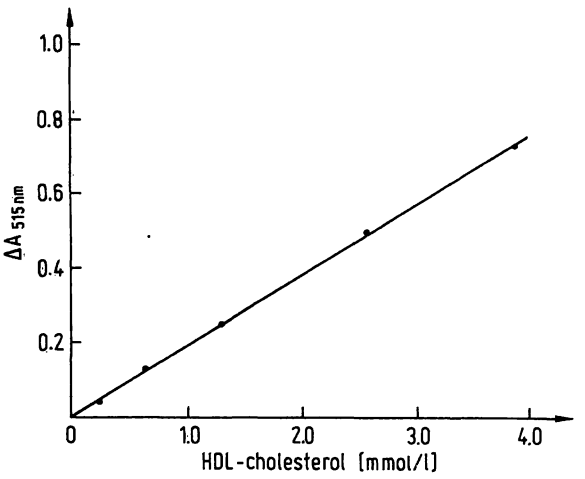

Fig. 3. Linearity of the fixed-time kinetic HDL cholesterol assay utilising 10 mmol/l 2,4,6-tribromo-3-hydroxybenzoic acid in the enzymic cholesterol reagent (duplicate assays).

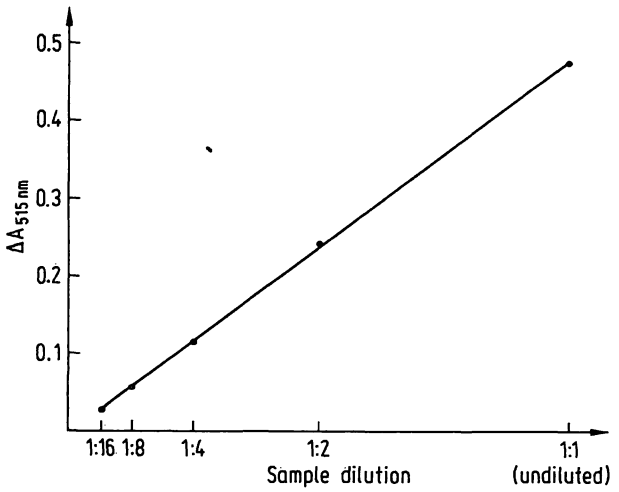

Fig. 4. Linearity of the fixed-time kinetic HDL cholesterol assay on serial dilutions of a human plasma containing 2.45 mmol/l HDL cholesterol (duplicate assays).

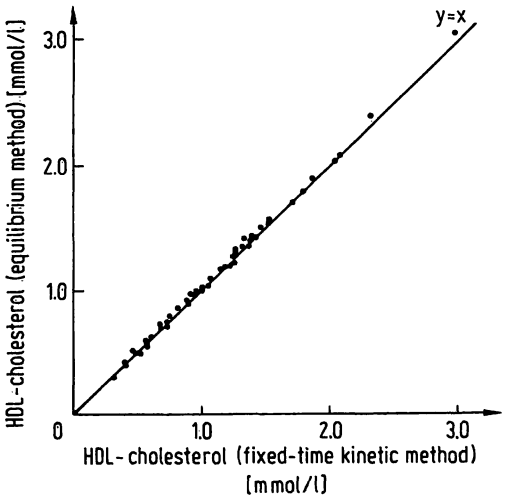

Fig. 5. Comparison of an equilibrium method (y) and the fixed-time kinetic HDL cholesterol method (x) in 48 human plasmas (slope = 1.011, axis intercept = 0.016 mmol/l, correlation coefficient = 0.9980).

Multiple analysis of the precinorm® L control serum produced mean values close to the manufacturer's value of 1.14 mmol/l (range = 0.96 to 1.32 mmol/l). The within-run precision using Precinorm L was represented by a coefficient of variation of 2.0% ($\bar{x}$ = 1.202 mmol/l, n = 20). The between-run precision was 2.6% ($\bar{x}$ = 1.180, n = 20). Analysis of a plasma pool with a low concentration of HDL cholesterol produced within-run and between-run coefficients of variation of 1.4% ($\bar{x}$ = 0.6763, n = 20) and 2.9% ($\bar{x}$ = 0.6902, n = 20) respectively. The within-run and between run precision of a plasma pool with a high concentration of HDL cholesterol were 1.1% ($\bar{x}$ = 1.6988, n = 20) and 1.6% ($\bar{x}$ = 1.7234, n = 20) respectively.

Interferences

The results of interferences by drugs, plasma metabolites or coloured constituents of blood are represented in table 2. Concentrations of the substances examined were added to a pooled specimen of human plasma. This plasma pool had a HDL cholesterol value of 1.24 mmol/l (mean of 10 assays) in the absence of any added substance.

Tab. 2. Interference of added substances (plasma concentrations of drugs usually found after administration of therapeutic doses in brackets).

Substances added, final concentration	Percentage of HDL cholesterol accounted for
Urea, 200 mg/l	99
Creatinine, 100 mg/l	99
Uric acid, 200 mg/l	99
Glucose, 4000 mg/l	98
Bilirubin, 50 mg/l	99
Bilirubin, 100 mg/l	98
Haemoglobin, 1000 mg/l	98
Haemoglobin, 2000 mg/l	97
Salicylate, 1000 mg/l (200 mg/l)	101
Gentisic acid, 30 mg/l (1 mg/l)	94
Gentisic acid, 50 mg/l	90
L-Dopa, 5 mg/l (1 mg/l)	90
L-Dopa, 10 mg/l	86
α-Methyldopa, 30 mg/l (1 mg/l)	79
α-Methyldopa, 50 mg/l	71
Ascorbic acid, 20 mg/l after 0 h at 20 °C	84
Ascorbic acid, 50 mg/l after 0 h at 20 °C	63
Ascorbic acid, 20 mg/l after 1 h at 20 °C	93
Ascorbic acid, 50 mg/l after 1 h at 20 °C	81
Ascorbic acid, 20 mg/l after 2 h at 20 °C	95
Ascorbic acid, 50 mg/l after 2 h at 20 °C	90
Ascorbic acid, 20 mg/l after 3 h at 20 °C	99
Ascorbic acid, 50 mg/l after 3 h at 20 °C	97

Discussion

The kinetic fixed-time technique for enzymatic analysis of total serum cholesterol was made possible by the use of cholesterol oxidase from a *Streptomyces* species instead of the enzyme from *Nocardia erythropolis* (8). The *Michaelis* constant (K_m) with respect to cholesterol of the *Streptomyces* enzyme can apparently be increased, by adding the competitive inhibitor, 3,4-dichlorophenol, to such an extent that the cholesterol oxidase reaction would follow first-order kinetics over a large range of cholesterol concentrations. By exploiting this inhibition, together with the very efficient cholesterol esterase, a kinetic cholesterol reagent was developed. In this kinetic system, the cholesterol oxidase reaction is the rate limiting step, so that the indicator reaction follows first-order kinetics (Boehringer Mannheim catalogue number 692 905 and 725 242).

Addition of 2,4,6-tribromo-3-hydroxybenzoic acid to this reagent to enhance the indicator reaction produces an increase in the sensitivity with respect to cholesterol so that a kinetic protocol may also be used to measure HDL cholesterol. Compared with the unmodified reagent, the method described in this report enables a three-fold increase in the absorbance differences (ΔA) of HDL cholesterol supernatants prepared with buffered polyethylene glycol 6000, a procedure which involves a two-fold dilution of plasma. The use of buffered polyethylene glycol 6000 to isolate HDL enables the clearing of most lipaemic plasmas which would otherwise cause an over-estimation of HDL cholesterol (11). The polyethylene glycol 6000 procedure is also accurate and efficient (12). Preliminary results also indicate that HDL supernatants prepared by the phosphotungstic acid/$MgCl_2$ (13) or dextran sulphate 500/$MgCl_2$ (14) β-lipoprotein precipitation methods may be assayed with the kinetic method on the Cobas-Bio analyser provided that it is programmed with a sample volume of 7 µl, instead of 15 µl.

The first-order kinetics of the HDL cholesterol kinetic method show a parallelism among the aqueous standards and the patient plasmas (fig. 2), indicating that the protocol described is suitable for routine estimation of patient HDL cholesterol values. Correlation between the kinetic and equilibrium methods was excellent and there was good agreement between the value obtained for the Precinorm® L control and the manufacturer's value. Linearity and precision are comparable to equilibrium methods (7, 11).

In contrast to the kinetic method for total serum cholesterol (8), in vitro addition of large amounts of substituted phenols such as *L*-dopa, methyldopa and gentisic acid to the plasma pool caused significant interference. However, the concentrations that these drugs attain in vivo would interfere negligibly (15). Interference from added ascorbic acid was minimised by leaving the HDL supernatant at room temperature for three hours before analysis. Ascorbic acid in solution rapidly oxidises in the presence of oxygen, especially at pH > 7.0 (16). In any case, a normal plasma ascorbic acid level of less than 20 mg/l should not interfere significantly and any higher levels due to excessive administration of vitamin C can be overcome by extending to 3 hours the interval between blood-taking and HDL cholesterol determination.

In conclusion, the present method involving 2,4,6-tribromo-3-hydroxybenzoic acid addition to the Boehringer Mannheim kinetic cholesterol reagent affords a faster, convenient analysis of HDL cholesterol than the corresponding equilibrium methods. Hence, the method is suitable for routine use in the automated laboratory. The method is also relatively cheap and has the potential of being used to measure the cholesterol in LDL and HDL subfraction isolates (HDL_2 and HDL_3).

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